Faculty of Resource Science and Technology

GENETIC RELATEDNESS STUDY OF *JATROPHA CURCAS* GENOTYPES USING RAPD MOLECULAR MARKERS

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Bachelor of Science with Honours
(Resource Biotechnology)
2013

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The project is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours

(Resource Biotechnology)

Department of Molecular Biology

Faculty of Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2013

ACKNOWLEDGEMENT

Alhamdulillah, grateful to Allah S.W.T for giving the strength and faith for me to finish this report. I would like to express my deepest appreciation to all those who provided me the possibility to complete this report. A special gratitude I give to our final year project supervisor, Mdm. Safarina bt Ahmad and Dr. Lesley Maurice Bilung, whose contribution in stimulating suggestions and encouragement, helped me to coordinate my project especially in writing this report. Furthermore I would also like to acknowledge with much appreciation the crucial role of the staff of Faculty of Resource Science and Technology (FRST) especially Mr.Dahlan and Miss Limjatai, who gave the permission to use all required equipment and the necessary materials to complete the final year project task. A special thanks goes to my team mate, Nur Atikah Anwar, Amirah Aqilah and Shirley Majin who help me to assemble the parts and gave suggestion about the task. For all the guidance and support from the postgraduate students, Mr. Nickson Chong (MSc. Student), Mr. Simon (MSc. Student) and Miss. Kathleen (MSc. student), I thank you. Many thanks go to course coordinator and the head of the department, Dr Samuel Lihan and Associate Prof. Dr Awg. Sallehin who have invested their full effort in guiding me in achieving the goal. My best friends, Farahiyah Senusi, Nur Arina Seruji, Nurul Atiqah Jamaludin, 'Inayat Zakiatuddinsyah and Hazirah Salleh, thanks for all the support and encouragement. Last but not least, to my dear coursemates who always be there to give their suggestions and supports, Noor Adzlina Abidin, Sitti Nur Janna, Umme Najehah Ahmad, Nurul Hidayah Ngadi, Nur Syahirah Othman and Siti Hajar, I thank you from the bottom of my heart.

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DECLARATION OF ORIGINAL WORK

This declaration is made on the July day of 1st 2013.

Student's Declaration:

I **NUR SYUHADAH BT MOHD GUSTI, 27708, FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY (FRST)** hereby declare that the work **Genetic Relatedness Study of *Jatropha curcas* Genotypes using RAPD Molecular Marker** is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

_____ _____

Date submitted Name of the student (Matric No.)

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LIST OF ABBREVIATIONS

AGE	Agarose Gel Electrophoresis
βME	β-mercaptoethanol
CIA	Chloroform-Isoamyl Alcohol
CTAB	Cetyltrimethylammonium Bromide
DNA	Deoxyribonucleic Acid
NaCl	Sodium Chloride
PVP	Polyvinylpolypyrrolidone
PCR	Polymerase Chain Reaction
RAPD	Random Amplified Polymorphic DNA
RNA	Ribonucleic Acid
TE	Tris-EDTA

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Genetic Relatedness Study of *Jatropha curcas* Genotypes using RAPD Molecular Marker

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ABSTRACT

Jatropha curcas is widely known as a source of high quality biodiesel. Due to the high demand of research on *J. curcas* accession, study of genetic relatedness of *J. curcas* accession from Sarawak (Lundu) to the other widely research *J. curcas* accession (Africa, Thailand and Indonesia) were conducted. The aim of this study was to assess the genetic relatedness of four different breeds of *J. curcas* (Africa, Thailand, Lundu (Sarawak, Malaysia) and Indonesia) by using RAPD marker. 10-mers primers were used to assess the genetic relatedness among the breeds. Jaccard formulation was used to calculate the similarity matrix. The RAPD-PCR analysis obtained shows high similarity matrix between Thailand and Indonesia (0.67) showing that both breeds come from a narrow genetic ancestor. Low similarity matrix of *J. curcas* of breed from Africa and Lundu to the other accession suggests that the breeds were not closely related. The successful revelation of genetic relatedness among the breeds indicates the effectiveness of RAPD profiling in the study. The outcomes of the study and the breeds are a good source for the development of excellent c *J. curcas* cultivars.

Keywords: *Jatropha curcas*, molecular marker, RAPD, genetic relatedness

ABSTRAK

Jatropha curcas (J. curcas) amat dikenali sebagai sumber biodiesel berkualiti tinggi. Oleh itu, kajian mengenai persamaan genetic antara J. curcas dari Sarawak (Lundu) terhadap Afrika, Thailand dan Indonesia adalah amat diperlukan bagi memenuhi permintaan tinggi ke atas kajian J. curcas. Objektif kajian ini adalah untuk menilai persamaan genetik pada empat baka yang berlainan (Afrika,Thailand, Lundu (Sarawak, Malaysia) dan Indonesia) dengan menggunakan penanda RAPD. Primer 10-mer telah digunakan untuk menilai perbezaan genetik antara baka.Formulasi Jaccard telah digunakan untuk mengira persamaan matriks. Analisis RAPD-PCR menunjukkan persamaan matriks yang tinggi antara Thailand dan Indonesia (0.67) memberi kumingkinan bahawa kedua baka ini berasal dari baka asal yang sama. Persamaan matriks yang rendah antara baka J. curcas dari Afrika dan Lundu ke atass baka yang lain memberi kemungkinan bahawa baka tersebut tidak berkait rapat antara satu sama lain. . Keberkesanan penanda molekul RAPD dalam kajian persamaan genetic dapat dibuktikan dalam kajian ini. Hasil kajian dan baka-baka ini dapat dijadikan sumber dalam pembagunan penghasilan kultivar J. curcas yang terbaik.

Kata kunci: Jatropha curcas, penanda molekul, RAPD, persamaan genetik

1.0 INTRODUCTION

Jatropha curcas belongs to Euphorbiaceae family and have many potential such as medicinal and biodiesel crop which has been recently recognized as a potential oil seed (Ahmadpour *et al*. 2010; Camellia *et al*., 2011). This plant has attracted such attention of the energy producers due to the potential for biodiesel (Camellia *et al*., 2011) and can be easily grown under marginal soil which requires little industrial input (Sunder, 2008). Thus, *J. curcas* has been introduced to many countries. There are various species of Jatropha and are widely distributed in the tropics and cultivated worldwide (Sujatha and Prabakaran, 1997).

J. curcas or known as Barbados nut and nowadays are more widely known as Physic nut are basically a poisonous shrub. *J. curcas* is also favored as the most suitable oil yields source of energy due to its various favourable attributes such as hardy nature, short gestation period, adaptability in wide range agro-climatic conditions, high oil recovery and quality of the oil produced. At the same time, throughout their life cycle (*J. curcas*), they improves the soil fertility (Pamidimarri *et al*., 2008).

According to Camellia *et al.* (2011), this plant is originated from the South/Central America and since then it spread to the Africa, Caribbean and warmer part of Asia such as Malaysia, Thailand, Indonesia and India. It is believed that *J. curcas* was introduced to Malaysia during the rule of Portuguese in 1511 from the Caribbean Island (Camellia et al., 2011). The survival of the plant depends on the adaptation of the plant itself especially plants that are newly introduced into a new environment. Therefore, natural selection will acts on random mutations in the population. Most of tree species produces offspring in order to survive. Throughout generations, those individuals who possess the most favourable traits will survive and

dominates the composition of population (Camellia et al., 2011). Due to this natural selection process, species has better adaptation to its environment and successful at reproduction (Brooker, 2009). There are three main effects that may happen to the population itself due to the environmental changes or to be specific on the adaptation of the plant to the environment. They are habitat tracking, genetic change or extinction. Out of these three, only genetic change brings about adaptation (Camellia *et al*., 2011). In a population, genetic changes occur when natural selection acts on the genetic variability of the population itself. Then, plants started to change their structure, physiology and its behavior in order to adapt. If they failed to adapt, they will not survive in the changing environment. There are various environmental factors that may causes changes in the deoxyribonucleic acid (DNA), which may alter the function and the DNA structure. Examples of the factors are; radiation, chemicals, abrupt changes in temperature, or other factors. The large majority of genetic variation within populations of certain species has its origin in mutations that occurs in single genes (Hartl and Clark, 1989).

Apart from the great potentials of *J. curcas* as a biodiesel crop, the full potentials of *Jatropha sp*. have not been realized (Raffi *et al*., 2012). This plant has adapted itself to wide range of environmental conditions and it is suggested that there are considerable amount of genetic diversity yet to be detected for potential realization. In Asia, there are wide researches conducted in order to obtain new information regarding on this beneficial plant especially in oil content yield and genetic diversity. Based on a research conducted by Akbar *et al*. (2010), given the results of oil content in percentages comparing *J. curcas* breed in term of seed oil composition among breeds from Malaysia, Indonesia and Thailand are 63.16, 61.36 and 64.23 respectively. It is concluded that Thailand gives the highest oil yield percentages. But,

Malaysia's *J. curcas* breed contains more unsaturated fatty acids (78.94%) compared to saturated fatty acids (21.05%) and shows that Malaysia's *J. curcas* breed has a bright potential in studying the genetic improvement of this plant itself (Salimon, 2008). In a recent research conducted by Salimon and Ahmed (2012), the study shows that percentages of oil yield from breed seed from Malaysia, Indonesia and India give readings of 33.7, 32.7 and 30.5 respectively. This research proved that Malaysia's *J. curcas* breed has the highest percentages of oil yield compared to the two other countries. Therefore, *J. curcas* breed from Thailand and Malaysia may possibly be the best breeds in studying the genetic improvement for the *J. curcas* itself and also the genetic diversity between the breeds.

Nowadays, various molecular markers techniques can be used to detect genetic differences. They had developed as powerful tools for diversity analysis and also establishing relationship between cultivars (Sharma *et al*., 2011). Molecular marker analysis offers an efficient alternative on this approach as genetic estimated and measured on the basis of genotype; not phenotype (Sharma *et al*., 2011). Furthermore, molecular markers analysis in genome studies enhances the efficiency of crop improvement (Pamidimarri *et al*., 2008).

The protocols to extract pure DNA from many plant species are available to date. However, published protocols available in the literature could not be successfully used in order to isolate high quality DNA from *J. curcas* (Pamidimarri *et al.,* 2008). *J. curcas* contain polysaccharides and polyphenols and pose a major problem in isolating a high quality DNA (Dhakhanamoorthy and Selvaraj, 2009). The differences between the genetic diversity and the relatedness of the samples can indicate the genetic differences and relatedness of the same species (*J. curcas*) but different breeds. Furthermore, the data are obtained from previous researches on *J. curcas* are mostly found in the India, Thailand, Indonesia and peninsular

Malaysia, but not in Sabah and Sarawak. To date, there are still not enough researches conducted on the genetic information for *J. curcas* found in Sabah and Sarawak. In addition, there is still no exact research on comparison of genetic diversity of *J. curcas* found in Sarawak to *J. curcas* located in Africa, Thailand and Indonesia. Therefore this problem leads to the question, whether *J. curcas* found in Sarawak are genetically different compared to *J. curcas* found in Africa, Thailand and Indonesia.

Thus, the objectives of this research were to extract and isolate high yield concentration of genomic DNA from the *J. curcas* leaves and to compare the genetic diversity of *J. curcas* from four different breeds (Africa, Sarawak (Lundu), Indonesia and Thailand) based on random amplified polymorphic DNA markers (RAPD).

2.0 LITERATURE REVIEWS

2.1 *Jatropha curcas*

Jatropha curcas (Euphorbiaceae family), also known as 'Jarak' in Malaysia, is an introduced plant in Malaysia which is grown for various purposes such as for its medicinal value and the seeds oil (Achten *et al*., 2010).The extracted oil of *J. curcas* is the most valuable end product and also an important source of biodiesel (Camellia *et al*., 2011). There are more than 200 species of Jatropha which are widely distributed in the tropic region and cultivated worldwide (Sujatha and Prabakaran, 1997). In addition, there are only a few Jatropha species can be found cultivated in Malaysia such as *Jatropha curcas, Jatropha gossypifolia, Jatropha podagrica, Jatropha multifida* and *Jatropha panduraefolia*. Among all the genus, *J. curcas* is the most known for its important in biodiesel yielding crop and is a multipurpose significant economic important plant.

2.1.1 Morphology

Jatropha genus has a wide morphologically diverse genus which comprising of shrubs, rhizomatous shrubs, herbs and small trees (Jubera *et al.*, 2009). Generally, *J. curcas* is known as Barbados nut or Physic nut. It is a perennial poisonous shrub that usually grows up to 5 m high in a short period of time (4-5 years).

J. curcas grow best on well drained soils with good aeration but they are also well adapted to marginal soils with low nutrient content soil as well as shallow fields and rocky

terrains. This plant can also withstand a long period of drought due to its low water requirement to grow (Camellia *et al*., 2011).

J. curcas fruit is green capsule shaped and contains 3 to 4 seeds (Figure 2.1). The seed are mature if the capsules colour changes from green to yellow. The seed contains unsaturated fatty acids and saturated fatty acids in term of oil yield. The oil is largely made up of oleic acid and linoleic acid. Furthermore, the seed also contains other chemical compounds; saccharose, stachyose, glucose, fructose, galactose, and protein (Nahar and Hampton, 2011).

The leaves of *J. curcas* are also varies in size. In general, the leaves are green to pale green, alternate to sub opposite, and three- to five-lobed with a spiral phyllotaxis (Figure 2.1). The leaves of *J. curcas* breeds of Africa, Thailand, Indonesia and Lundu had similar leaf features. The leaves were mostly comprises of 5 lobes (Figure 2.2)

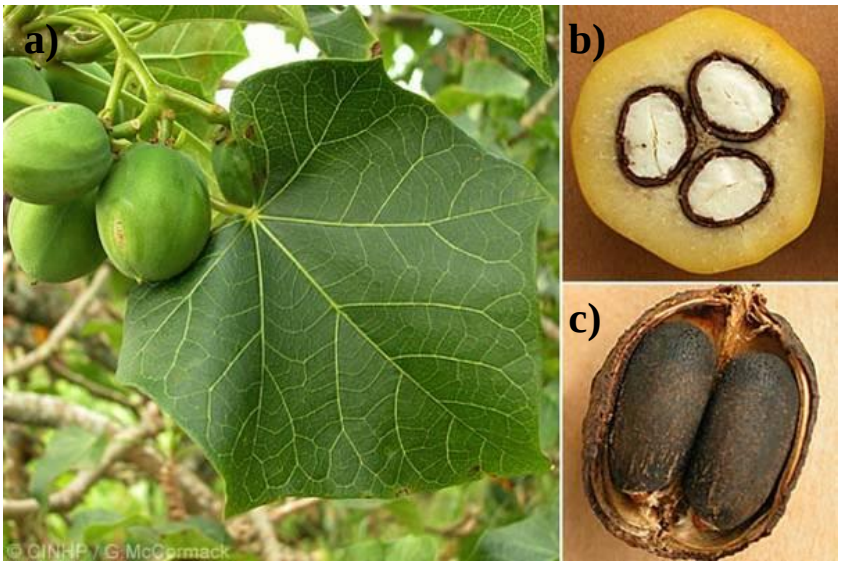

Figure 2.1: Morphology of *Jatropha curcas* leaf, fruit and seed; (a) *J. curcas* leaf and fruit; (b) Seed of *J. curcas* (dissection); (c) Seed of *J. Curcas* (Adapted from http://biomechanism.com/natural-pain-relief-from-a-poisonous-shrubcalled-jatropha-curcas/)

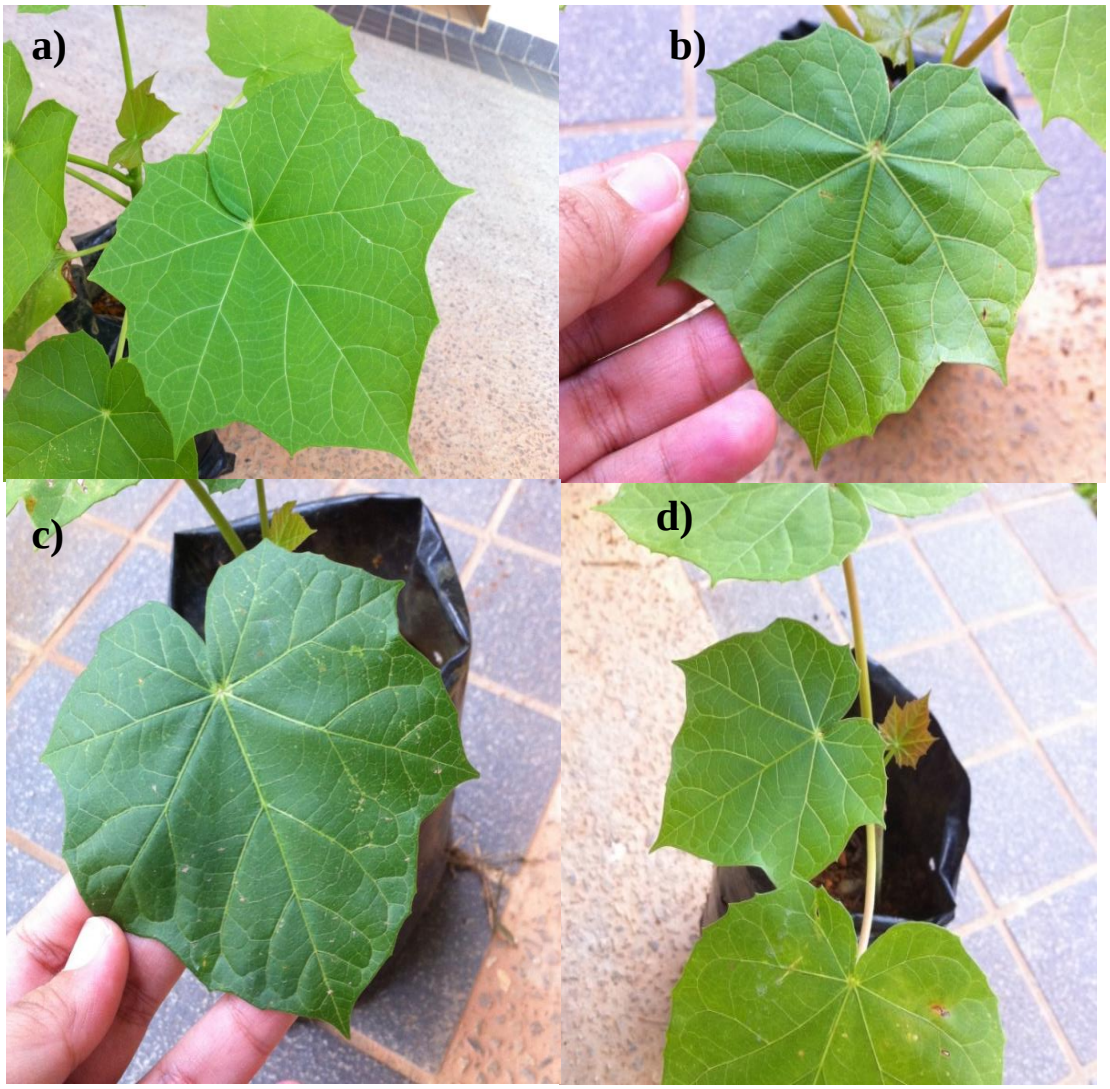

Figure 2.2: The leaf structures of *J. curcas* comprises of 5 lobes. (*J. curcas* breed samples of a) Africa; b) Indonesia; c) Lundu; d) Thailand.

2.1.2 Origin

The origin of this plant is reported from the Mexico and tropical South America and it is also reported that this plant is introduced into Asia and Africa by the Portuguese as an oil yielding plant (Jubera *et al.*, 2009). According to Camellia *et al.* (2011), *J. curcas* was introduced to Malaysia during the rule of Portuguese in 1511 from the Caribbean island.

Jatropha sp. had been used as an energy source for aircraft at the time Japanese conquered Malaysia in the year of 1940 until 1945 (Bernama, 2007). It was vaguely reported regarding the forms of propagules when *J. curcas* was firstly introduced in Malaysia.

2.1.3 Variability

Variability is important for selection and it is needed to detect and document the amount of variation exist within and between populations (Jubera *et al.*, 2009). *J. curcas* is cross pollinated and resulting in a high degree of variation. From a study done by Henning (2009), the highest genetic differences of *J. curcas* were found in the Central America and the genetic profile in other parts found throughout the world is similar. In a previous study done by Camellia *et al.* (2012), low amount of genetic diversity among 16 *J. curcas* grown throughout the Peninsular Malaysia and Sabah (Johor, Selangor, Kedah, Pahang, Kelantan, Terengganu and Sabah). The Jaccard's coefficient of similarity obtained from the study ranged from 0.72 to 1.00. The lowest similarity matrix was found between accessions of Kelantan and Selangor which was 0.72. Based on the results obtaned, Camellia *et al.* (2012) suggests *J. curcas* in Malaysia comes from the same source or due to the low number of markers used in the study.

2.2 DNA extraction

With the application of molecular techniques in plant diversity conservation becoming increasingly popular, the isolation of impact, high-molecular mass genomic DNA becomes an important pre-requisite (Dhakshanamoorthy and Selvaraj, 2009). However, species of Jatropha including *J. curcas* contains polysaccharides and polyphenol posed a major problem in the isolation and extraction of high quality DNA. Although several protocols were used for isolation of genomic DNA in *J. curcas*, including Ganesh Ram *et al.*, (2008); Ranade *et al.*, (2008); Pamidiamarri *et al.*, (2008) and Basha and Sujatha (2007), all of these protocols use expensive and toxic chemicals liquid nitrogen.

The modified CTAB extraction protocol introduced by Dhakshanamoorthy and Selvaraj, (2009) emit the use of liquid nitrogen by replacing it with absolute alcohol as the fixing solution. Fixing solution is used as to break down the cell wall of the plant samples. The result outcome from the study done by Dhakshanamoorthy and Selvaraj, (2009) showed the quality of the obtained DNA yield were similar to results reported by the other protocols (Sharma et al., 2003; Chandra and Tewari, 2007; Khan et al., 2004). Taking into consideration the limitation of liquid nitrogen in less equipped laboratory, method emits the use of liquid nitrogen would be very helpful and economical.

2.3 Molecular markers

Using molecular marker based fingerprinting, plant species can be distinguish using a small amounts of DNA and therefore, the genetic variation within or between populations can be identified (Ganesh *et al*., 2008). Molecular markers are not influenced by the condition of the environment typically and therefore are suitable to be used in describing the patterns of genetic variation among plant populations (Jubera *et al.,* 2009). Furthermore, to date, number of studies on *J. curcas* in genetic research is increasing due to the advancement of molecular marker itself.

There are various approaches are available nowadays. Examples are restriction fragment length polymorphism (RFLP), Inter-simple sequence repeats (ISSR), amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD).

2.3.1 RAPD fingerprinting

Among all the techniques listed, RAPD technique is the most widely used in *J. curcas* genetic studies (Sharma *et al.,* 2011). It is not costly and the rapid method does not require any information regarding the genome of the plant.

Based on a research done by Sharma *et al.* (2011), RAPD techniques are technically the simplest, less expensive, fast and high infrastructure is not required to start with. Only a small amount of genomic DNA is required to obtain the results of the DNA analysis. Furthermore, high level of polymorphism can be produced and may facilitate more effective

diversity analysis in plants (Jubera *et al*., 2009). This technique is widely used by the researchers in obtaining the gene diversity of Jatropha plant.

Compared to other molecular fingerprinting methods, RAPD fingerprinting gives higher constituency compared to aloenzyme and AFLP technique (Liu and Furnier, 1993). According to Gupta *et al.* (2008), RAPD markers were more efficient than the ISSR assay in detecting polymorphism, as they detect 84.26% as compared to 76.54 for ISSR markers. Thus, this technique is suitable and reliable to be conducted in genetic relationship analysis even though the background of the genome studied is not fully identified or yet fully identified.

In standard RAPD fingerprinting, the total genomic DNA amplification requires the utilization of short synthetic oligonucleotides which is 10 bases long of random sequence as primers. This is done under the low annealing temperature by PCR (Bardakci, 2001). The products are normally separated on the agarose gel and stained with ethidium bromide. There is no prior knowledge needed on the genome in conducting the RAPD analysis (Bardakci, 2011).

During the thermal cycle with suitable annealing temperature, the random sequences oligonucleotides primers will bind to several of the priming sites on the complementary sequences. Later, discrete DNA products will be produced if only these priming sites are within an amplifiable distance between sites (Bardakci, 2001). Nucleotide variation between the different sets of the templates DNA will resulting either the presence or absence of bands due to changes in priming sites (Bardakci, 2001).

3.0 MATERIALS AND METHODOLOGY

3.1 Plant materials

A total of 25 seeds of *J. curcas* from the accession obtained (Africa, Thailand, Indonesia and Lundu) were set to germinate on soil under natural condition. Germination time for scarified *J. curcas* seeds is between 10 – 50 days and it depends on temperature and environment. Fresh middle stage leaves and young leaves were collected (Figure 3.1). Then, the leaves were washed using tap water to wash impurities before performing 70% ethanol wipe.

Figure 3.1: *Jatropha curcas* leaves. The leaves are varies in size. There are three- to five-lobed with a spiral phyllotaxis. (Sample of *J. curcas* of breeds of Africa).

3.2 Reagents and stock Buffers

Reagents and buffers used in this study are listed in Table 3.1. The preparation of the reagents and buffers were according to Appendix A.

Table 3.1: Buffers and solution used for DNA isolation

Buffer	**Contents**
CTAB extraction	1 ml CTAB extraction buffer; pre-heated at 65 °C), 50 µl of Tris-EDTA
Chloroform Isoamyl Alcohol (CIA)	24:1, (v/v)
Iso-propanol	Cold; (-20°C); 2/3 of the volume; (~600 µl)
Ethanol	70% and absolute
Polyvinylpolypyrrolidone (PVP)	2% (added into CTAB buffer before extraction)
B-mercaptoethanol (βME)	2% (added into CTAB buffer before extraction)

3.3 DNA Extraction

Total genomic DNA was extracted from the young leaves and middle stage leaves from each breed following the CTAB method with minor modifications (Dhakshanamoorthy and Selvaraj, 2009). The leaves were weighed 0.5-1 g and cut into small pieces. The tissues were incubated in approximately 200 ml of absolute ethanol for 60 minutes (Figure 3.2).

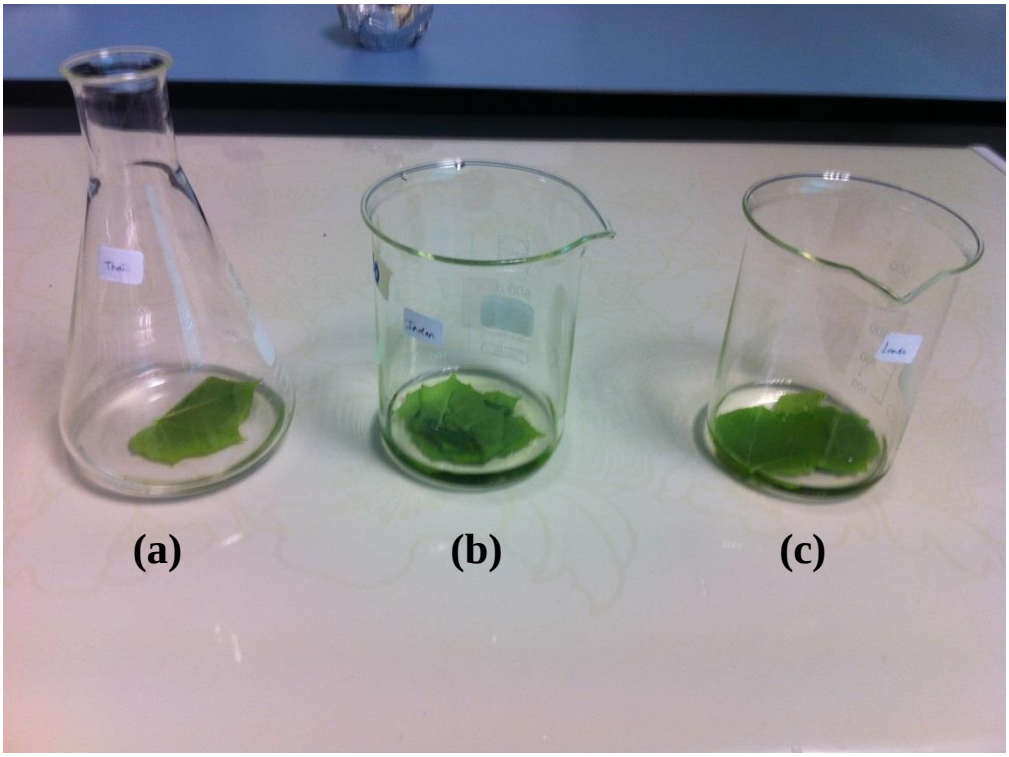

Figure 3.2: The *J. curcas* leaves were incubated in absolute ethanol for 60 minutes (room temperature). The *J. curcas* samples were breeds of a) Thailand; b) Indonesia; c) Lundu.

The leaves were then left to air dried to dry completely allowing alcohol to evaporate. The leaf tissues were grounded in a preheated at 65°C CTAB extraction buffer. Then it was transferred as fast as possible into a 1.5 ml eppendorf (EP) tube with the help of spatula. The samples were incubated for 60 minutes at 65°C and mixed gently by inverting the tube every few minutes. The tubes then removed from the water bath and allowed to cool to ambient. 200 µl of CIA was added and gently mixed. The samples then were centrifuged at 13,000 rpm for 5 minutes to obtain supernatant (protein and polysaccharide removed). The aqueous phase (the top phase) was transferred to a clean 1.5 ml EP tube by using a wide-bore pipette or blue tip. Then 500 µl of CIA was added and was mixed gently to a single phase. The samples were then centrifuged at 13,000 rpm in order to obtain the supernatant. The tubes then again were retrieved from the centrifuge machine. The aqueous phase was transferred into a clean 1.5 ml

EP tube. Then, 2/3 of the volume (~600 µl) of cold isopropanol (-20 °C) was added and gently mixed by inverting the tube to precipitate the nucleic acids. The tubes then were stored in the freezer (-20 °C) for 1 hour. The samples then were centrifuged at 13,000 rpm for 10 minutes to obtain the DNA pellet. The samples were carefully retrieved from the centrifuge machine. The supernatant was gently poured carefully to make sure the pellet is not lost. Then, 1 ml of 70% alcohol was added and the pellet will be rinsed by tapping the tube gently. The DNA pellet then was dissolved in 500 µl double distilled water. 50 µl of 3M sodium acetate and 1000 µl of absolute alcohol were added into the samples. Then, the samples were stored in a -20 °C freezer for 1 hour. The samples were then centrifuged at 13,000 for 10 minutes. The supernatant was discarded and the pellet will be left to be dry (by inverting the tube) to eliminate the alcohol completely. The dried pellet then was resuspend in 50 µl of TE (Tris-EDTA) buffer (without RNase A).

3.4 DNA Quantification

The quantity (concentration) and quality of DNA was determined by using spectrophotometer at wavelengths 260 and 280 nm. Determination of the total DNA quantity was calculated based on the value of absorbance at a wavelength of 260 nm. At 260 nm = 1.0 equivalent amount of DNA is 50 ug/mL (Prayitno & Nuryandani, 2011). λ DNA quality is considered good if the value of A260/A280 approaching 1.8 to 2.0. The concentration and the purity of the DNA was also checked by running the samples on 1.0% agarose gels along with Fermentas , GeneRuler ™ as a DNA marker.